EPR Detection of Guanine Radicals in a DNA Duplex under Biological Conditions: Selective Base Oxidation by $Ru(phen)_2dppz^{3+}$ Using the Flash-Quench Technique

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Continuous-wave X-band EPR spectroscopy has been employed in examining the guanine radical within a DNA duplex at ambient temperature using the flash-quench technique. Guanine was selectively oxidized by DNA-bound $[Ru(phen)_2dppz]^{3+}$ (dppz = dipyridophenazine, phen = 1,10-phenanthroline) generated in situ by photolysis in the presence of $[Co(NH_3)_5Cl]^{2+}$ as the oxidative quencher. An EPR signal centered at g_{iso} = 2.0048 is observed in experiments with poly(dG-dC) as substrate. Comparable signals are also detected with a 13-mer oligonucleotide duplex containing only one guanine base and with calf thymus DNA, but no signal is observed with poly(dA-dT) or poly(dI-dC). These observations reflect the base selectivity of the reaction in forming the guanine radical. With ruthenium hexaammine as oxidative quencher, no signal is observed, while, with methyl viologen, a strong signal with hyperfine pattern is seen, characteristic of the reduced viologen radical and indicating that $[Ru(phen)_2dppz]^{3+}$ was generated. The guanine radical signal, once formed upon continuous irradiation in argon-saturated aqueous buffer solution (pH 7), decays with a half-life of 30 s, but vanishes instantaneously in the dark or upon introduction of oxygen. Spin trapping experiments with *N-tert*-butyl-α-phenylnitrone substantiate the selectivity in generating the guanine radical; in the presence of poly(dG-dC), calf thymus DNA, the 13-mer oligonucleotide but not with poly(dA-dT) and poly(dI-dC), the detected nitroxide EPR signals are the same with g_{iso} = 2.0059, $\langle a_N \rangle$ = 15.05 G, and $\langle a_H \rangle$ = 3.11 G. Upon titration of the ruthenium intercalator into poly(dG-dC), the signal intensity increases smoothly as the [base pair]/[intercalator] ratio decreases from 100 to 25, at which point the signal intensity decreases markedly; this result may be an indication of an antiferromagnetic exchange interaction between guanine radicals. Indeed, using the flash-quench technique, EPR spectroscopy of guanine radicals within DNA now will permit the evaluation of how radicals within the DNA base stack may be coupled under biological conditions.

Introduction

A critical issue with respect to understanding the chemistry of mutagenesis and carcinogenesis involves the elucidation of how radicals interact within DNA. During the past decade, there have been substantial efforts made to elucidate how electrons and electron holes are transported through DNA.[1] In our laboratory, we have most recently systematically probed photoinduced long-range electron transfer through DNA by fluorescence quenching and transient absorption spectroscopy,[2] as well as by electrochemical[3] and biochemical[4] methods. From these different experiments, it has become clear that long-range electron transfer through DNA depends sensitively upon the intimate π-stacking of the electron donor and acceptor as well as the intervening bases. On the basis of this facility of charge transport through the DNA duplex, both oxidative damage to DNA and the oxidative repair of a thymine dimer in DNA have been demonstrated from a remote position on the helix.[4,5] Indeed, oxidative damage to DNA has been observed over a distance of 200 Å[4] and has been shown to depend on intervening sequence and to be modulated by DNA-bound proteins.[6]

EPR spectroscopy serves as a valuable tool in characterizing radicals and how they react and interact along the DNA helix. Many experiments have been conducted at low temperature with bound or intrinsic radicals within the DNA double helix being generated using ionizing radiation.[7−10] These studies have provided a broad range of estimates for radical migration in DNA, anywhere from 1 to 200 base pairs, but the positions and distances traveled by discrete radicals were not known with any certainty. It is interesting that some studies employing intercalators as spin traps have suggested charge migration over >25 Å.[8] For example, EPR measurements of the reduced form of the intercalating antitumor agent daunomycin provided evidence for the disproportionation of this radical over 100 base pairs.[10] However, fewer studies have been carried out which monitor base radicals in DNA directly. EPR spectra have been reported for the individual nucleic acid bases and nucleotides in solution at room temperature,[11] but EPR spectra of duplex DNA were obtained only in frozen solution or in the solid state at low temperature and as superpositions of signals for different base and/or sugar radicals.[12,13] Indeed, many of these experiments have focused on the generation of radicals using reductive chemistry.

A useful method previously applied in our laboratory to generate guanine radicals within the DNA duplex is the flash-quench technique (Scheme 1).[14] The flash-quench cycle is initiated by visible light, which excites $[Ru(phen)_2(dppz)]^{2+}$ (dppz = dipyridophenazine, phen = 1,10-phenanthroline) in-

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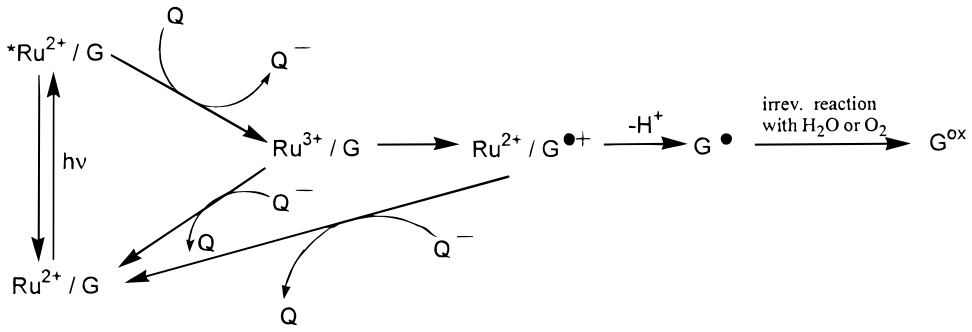
SCHEME 1: Flash-Quench Cycle



tercalatively bound to DNA. The excited Ru^{2+} complex ($*Ru^{2+}$) is then quenched by electron transfer to a nonintercalated electron acceptor (Q), e.g. $[Co(NH_3)_5Cl]Cl_2$, $[Ru(NH_3)_6]Cl_3$, or MV^{2+} (methyl viologen), to form an intercalated $[Ru(phen)_2(dppz)]^{3+}$ complex, which is a powerful ground-state oxidant (1.6 V).[15] $[Ru(phen)_2(dppz)]^{3+}$, once generated, has a thermodynamic potential which is sufficient to oxidize guanine (1.3 V)[16] to the guanine radical cation ($G^{\bullet+}$) within DNA. The guanine radical cation is then rapidly deprotonated,[17] yielding the neutral guanine radical ($G^{\bullet}$), which has been identified using transient absorption spectroscopy.[14] The neutral guanine radical may then react irreversibly with water and/or oxygen to form stable oxidized products, G^{ox}, which can be analyzed further using biochemical methods. An advantage of the flash-quench methodology, therefore, is that it leads to the generation of guanine radicals within the intact DNA duplex in aqueous buffered solution at pH 7 and ambient temperature.

Here we apply EPR spectroscopy to probe the guanine radical in DNA duplexes formed by electron transfer using the flash-quench technique. We observe, using EPR spectroscopy, that the flash-quench technique generates guanine radicals in heteropolymeric DNA in a remarkably selective fashion in aqueous solution. Spin-trapping experiments further support the assignments. We can therefore now apply EPR spectroscopy to the characterization of the guanine radical within the DNA duplex under biological conditions and in the absence of other interfering radicals.

Experimental Section

Materials. Poly(dG-dC), poly(dI-dC), and poly(dA-dT) were purchased from Pharmacia Biotech and were exchanged into standard buffer (10 mM tris HCl, pH 7) by ultrafiltration (Amicon) prior to use. The single-stranded oligonucleotides were prepared on an Applied Biosystems 394 DNA synthesizer, using standard phosphoramidite chemistry, and then purified by reversed phase high-performance liquid chromatography (Hewlett-Packard Series 1050). Synthetic duplexes were hybridized by slow cooling (90−20 °C) of equal concentrations of complementary strands in aqueous buffered solution (10 mM tris HCl, pH 7). $[Ru(phen)_2(dppz)]Cl_2$ was prepared as described previously.[18] $[Co(NH_3)_5Cl]Cl_2$, $[Ru(NH_3)_6]Cl_3$ and methyl viologen dichloride were purchased from Aldrich and used as received.

Sample Preparation. Samples contained 0.07 mM $[Ru(phen)_2(dppz)]Cl_2$, 0.7 mM quencher, and 7 mM base pairs of poly(dG-dC), poly(dI-dC), or poly(dA-dT) in an aqueous buffer of 10 mM tris HCl (pH 7) using extinction coefficents of $\epsilon_{440} = 21\,000\ M^{-1}\ cm^{-1}$, $\epsilon_{254} = 8400\ M^{-1}\ cm^{-1}$, $\epsilon_{251} = 6900\ M^{-1}\ cm^{-1}$, and $\epsilon_{262} = 6600\ M^{-1}\ cm^{-1}$ for $[Ru(phen)_2(dppz)]Cl_2$, poly(dG-dC), poly(dI-dC), and poly(dA-dT), respectively. The concentrations for the sample with the synthesized DNA oligomer were 7 mM DNA duplex, 0.7 mM $[Ru(phen)_2(dppz)]^{2+}$, and 0.7 mM $[Co(NH_3)_5Cl]^{2+}$ quencher using extinction coefficents ϵ_{260} of 12300, 7400, 15000, and 6700 $M^{-1}\ cm^{-1}$ for dG, dC, dA, and dT, respectively. The solutions were saturated with argon and transferred into argon flushed quartz flatcells (Wilmad, WG-808).

EPR Setup. EPR spectra were recorded on a continuous-wave X-band EPR spectrometer (EMX, Bruker) using a TM_{102} cylindrical cavity with a grid of 5 slits of 0.4 cm width. The light source used was a 300 W Xe-arc lamp (Varian Eimac Division, Light R300-3) powered by an Illuminator Power Supply (Varian Eimac Division, model PS 300-1). The light was passed through an IR- (water) and UV-filter ($\lambda < 340$ nm) and focused on the grid in the cavity. Spectra were recorded during irradiation. The *g*-values were determined by comparison with 3-Carboxy-PROXYL as internal standard ($g_{iso} = 2.007$). The simulations were done with "Simfonia" software (Bruker). Line width effects were simulated according to $\Delta = a + bM_I + cM_I^2$, where Δ corresponds to the line width, *a*, *b*, and *c* are adjustable parameters, and M_I is the quantum number for the *z* component of the nuclear spin angular momentum.[19]

Results and Discussion

Detection of Guanine Radical in Poly(dG-dC). EPR experiments were initially carried out using poly(dG-dC) as substrate with a variety of quenchers. These flash quench reactions have been shown previously to be tunable based upon quencher selection; the yield of guanine radical and irreversible guanine damage are seen to increase for the series of quenchers $[Ru(NH_3)_6]^{3+} < MV^{2+} < [Co(NH_3)_5Cl]^{2+}$, likely reflecting the stability of the quencher in the reduced form and its susceptibility to back electron transfer.[14]

EPR spectra of poly(dG-dC), irradiated in the presence of $[Ru(phen)_2dppz]^{2+}$ and each of the three quenchers $[Co(NH_3)_5Cl]^{2+}$, MV^{2+}, or $[Ru(NH_3)_6]^{3+}$, are shown in Figure 1. A signal without resolved hyperfine structure was obtained with $[Co(NH_3)_5Cl]^{2+}$ as quencher. The low intensity of the signal prompted the increase in modulation amplitude to 8 G and hence a loss of any detectable hyperfine structure. Importantly, no EPR signal was detected in the absence of light or upon omitting any one of the three components, DNA, intercalator, or quencher; addition of the missing constituent led to the immediate rise of the signal. The equivalent signal was obtained for poly(dG-dC) irradiated in the presence of $[Ru(phen)_2dppz]^{2+}$ and $[Co(NH_3)_5Cl]^{2+}$ using 0.1 M phosphate buffer under the same conditions, showing that the signal is not associated with radicals from the tris buffer.[20]

With MV^{2+} as quencher instead of $[Co(NH_3)_5Cl]^{2+}$, the strong EPR signal with resolved hyperfine structure, shown in Figure 1B, was obtained. By comparison with published spectra,[21] this signal is clearly assigned to the more stable $MV^{\bullet+}$ radical. In this case, the signal did not decay measurably over several hours.

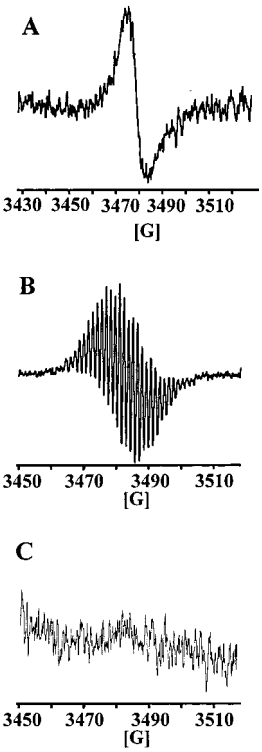


Figure 1. EPR signals obtained with different quenchers during continuous irradiation with visible light. Samples, prepared and measured under argon, contained 7 mM poly(dG-dC), 0.07 mM [Ru(phen)$_2$(dppz)]$^{2+}$, and 0.7 mM quencher (A) [Co(NH$_3$)$_5$Cl]$^{2+}$, (B) methyl viologen, and (C) [Ru(NH$_3$)$_6$]$^{3+}$ in 10 mM tris buffer, pH 7. For (A), the signal width $\Delta = 45$ G, half width at half-height 8 G, $g_{iso} = 2.0048$. For (B), $\langle a_N \rangle = 4.23$ G, $\langle a_H(CH_3) \rangle = 3.99$ G, $\langle a_{H2} \rangle = 1.33$ G, $\langle a_{H3} \rangle = 1.57$ G, $g_{iso} = 2.0030$. For (C) no EPR signal is observed in the presence of the [Ru(NH$_3$)$_6$]$^{3+}$ quencher. EPR settings: $T = 295$ K; microwave frequency 9.80 GHz; modulation frequency 100 kHz; modulation amplitude (A) 8 G, (B) 0.2 G, and (C) 8 G; microwave power 10 mW; conversion time 20.9 ms; receiver gain 6.3×10^5; number of scans (A) 1, (B) 5, and (C) 10.

Again, this signal could not be detected in the dark, or upon irradiation but omitting any of the sample components, or upon exposure to oxygen. Use of [Ru(NH$_3$)$_6$]$^{3+}$ as quencher failed to afford any detectable signal (Figure 1C).

The requirements for all components in addition to light to form the MV$^{\bullet+}$ radical as well as the radical giving rise to the EPR signal in Figure 1A are consistent with the generation of these radicals in the context of the flash-quench cycle. Furthermore, since MV$^{\bullet+}$ is generated by electron transfer from the excited Ru^{2+} intercalator to the MV^{2+} quencher, the detection of the MV$^{\bullet+}$ hyperfine pattern indicates also the successful formation of the corresponding Ru^{3+} intercalated oxidant. Once formed, the intercalated Ru^{3+} complex is thermodynamically capable of oxidizing guanine but not cytosine bases.[22]

Thus we assign the EPR spectrum shown in Figure 1A to that of the guanine radical (G$^{\bullet}$) within the DNA duplex, formed by electron transfer to Ru^{3+} and subsequent deprotonation of the guanine radical cation (p$K_a = 3.9$)[16] at the experimental pH of 7.0. The g_{iso} value of ≈ 2.0048 for G$^{\bullet}$ that we observe is close to the g_{iso} value of $\approx$2.0045 for a single line signal detected in X-ray-irradiated dry DNA at low temperature, after warming the sample to 300 K and refreezing to eliminate less stable radicals.[23]

As illustrated in Figure 2, with constant irradiation, the signal observed using 0.7 mM [Co(NH$_3$)$_5$Cl]$^{2+}$ as quencher decayed with a half-life of 30 s. This decay of G$^{\bullet}$ is likely responsible

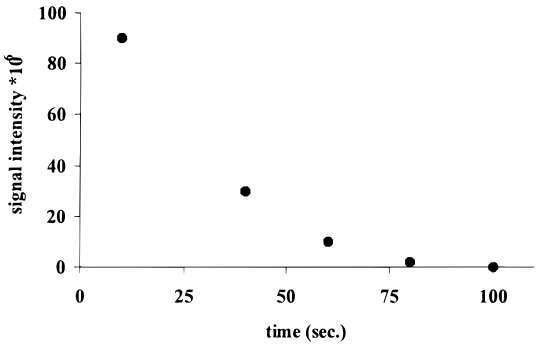


Figure 2. Decay of the guanine radical signal, measured on the basis of double integrated signal intensities. Conditions are the same as in Figure 1A, with the decay measured during illumination.

for the weaker intensity of the high-field peak compared to the low-field peak of the signal (factor of 1.3), since we can quantitatively account for the asymmetry in peak shape on the basis of the decay kinetics of the radical during the course of scanning. Additionally, the signal diminished rapidly upon removal of light or upon introduction of oxygen. Increased concentrations of quencher led to a more rapid decay of the signal; perhaps this reflects subsequent reactivity of the G$^{\bullet}$ with the quencher or its reaction products. Earlier transient absorption measurements showed that G$^{\bullet}$ persisted at least to the millisecond regime.[14] The sensitivity of the EPR signal to oxygen indicates also efficient radical quenching by oxygen.

It is also understandable that, with MV^{2+} as quencher, the G$^{\bullet}$ signal is not seen. Because of the superposition of any G$^{\bullet}$ signal with the strong and long-lived MV$^{\bullet+}$ spectrum, it was not possible to deconvolute any underlying signal which could be attributed to the G$^{\bullet}$. Indeed, the signal is expected to be 1 order of magnitude weaker than the signal obtained with the [Co(NH$_3$)$_5$Cl]$^{2+}$ quencher on the basis of comparisons with transient absorption studies and gel electrophoresis experiments using these two quenchers.[14] Using [Ru(NH$_3$)$_6$]$^{3+}$ as quencher, the yield of irreversible oxidation products of guanine was 3 orders of magnitude lower, and therefore, we might expect a G$^{\bullet}$ concentration that is too low to be detected by X-band EPR; in fact with this quencher no signal was observed (Figure 1C).

EPR Experiments with Poly(dA-dT) and Poly(dI-dC). Control experiments were carried out using poly(dA-dT) and poly(dI-dC) as substrate, irradiated in the presence of [Ru(phen)$_2$dppz]$^{2+}$ and each of the three quenchers. These experiments represent more appropriate controls than those carried out for [Ru(phen)$_2$dppz]$^{2+}$ irradiated in the presence of quencher but without DNA, because [Ru(phen)$_2$dppz]$^{3+}$ is not formed to an appreciable extent without DNA owing to the exceedingly short excited-state lifetime (250 ps) for [Ru(phen)$_2$(dppz)]$^{2+}$ in water; hydrogen bonding of H$_2$O to the phenazine nitrogens leads to rapid quenching of the nonintercalated [Ru(phen)$_2$(dppz)]$^{2+}$ excited state.[24]

EPR experiments using poly(dA-dT) and poly(dI-dC), irradiated in the presence of [Ru(phen)$_2$dppz]$^{2+}$ and each of the three quenchers [Co(NH$_3$)$_5$Cl]$^{2+}$, MV^{2+}, or [Ru(NH$_3$)$_6$]$^{3+}$, were therefore carried out. With poly(dA-dT) and poly(dI-dC), there was no signal detected using either [Co(NH$_3$)$_5$Cl]$^{2+}$ or [Ru(NH$_3$)$_6$]$^{3+}$ as quencher. However, with MV^{2+}, we again observed the characteristic MV$^{\bullet+}$ spectrum, which could not be detected in the dark or upon omitting one of the sample components. The generation of MV$^{\bullet+}$ therefore indicated the formation of the intercalated Ru^{3+} oxidant bound to poly(dA-dT) and poly(dI-dC).

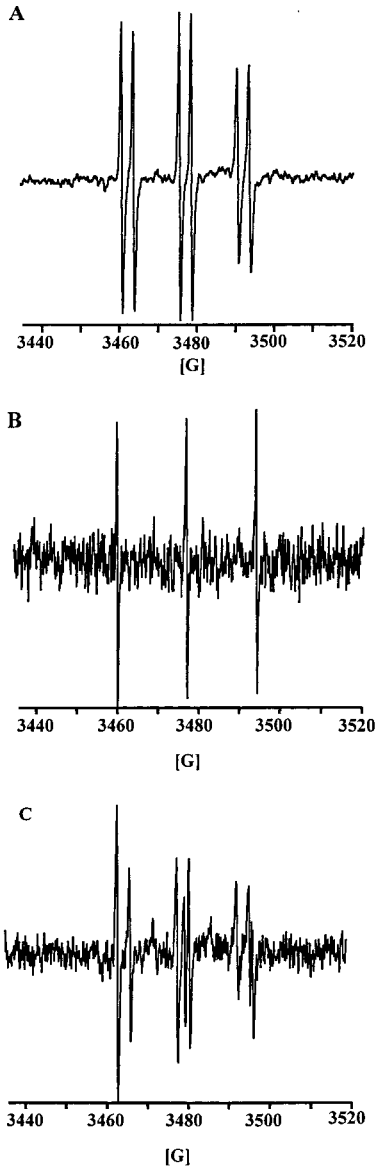
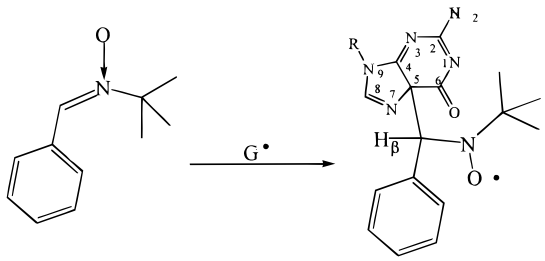


Figure 3. EPR spectra of the spin-trapped G• in the dark (A) after 1 min irradiation, (B) after a second irradiation for 10 s, and (C) after 10 h in the sunlight. Substrate DNA concentrations were 7 mM poly(dG-dC), 0.7 mM $[Co(NH_3)_5Cl]^{2+}$, 0.07 mM $Ru(phen)_2(dppz)]^{2+}$, and 0.7 mM PBN in 10 mM tris buffer, pH 7. EPR settings: $T = 295$ K; microwave frequency 9.80 GHz; modulation frequency 100 kHz; modulation amplitude 0.7 G; microwave power 10 mW; conversion time 20.9 ms; receiver gain 2×10^5; number of scans 3.

The absence of any EPR signal with $[Co(NH_3)_5Cl]^{2+}$ as quencher was also significant. This result shows clearly that no adenine, thymine, cytosine, inosine, sugar, or metal complex radicals are formed using the flash-quench technique, or if they are generated, they appear to be EPR silent. The absence of a signal from the generated Ru^{3+} intercalator is likely due to a fast electron spin relaxation caused by orbital degeneracy of the singly occupied molecular orbital of the complex. There is also no EPR signal detected for the reduced $[Co(NH_3)_5Cl]^{2+}$, because the Co(II) complex degrades irreversibly to the aquated d^7 complex, which also possesses an electron spin relaxation time which is too short to yield a detectable EPR signal under these conditions. Clearly, then, the signal in Figure 1A, with poly(dG-dC) as substrate, must correspond solely to the guanine radical. Additional explanations and implications of this base selectivity are considered in more detail below.

SCHEME 2: Proposed Spin-Trapping Reaction of G• with PBN



Spin-Trapping of the Guanine Radical. The most commonly used reagent to trap DNA or nucleotide radicals is 2-methylnitrosopropane (MNP),[26] but under our experimental conditions, we observed its photodecomposition to di-*tert*-butyl nitroxide.[27] Trapping experiments were therefore carried out using an alternate reagent, *N*-*tert*-butyl-α-phenylnitrone (PBN).[28]

After irradiation of the solution containing poly(dG-dC), $[Ru(phen)_2dppz]^{2+}$, $[Co(NH_3)_5Cl]^{2+}$, and 1 mM PBN for 1 min, the spectrum shown in Figure 3A was recorded in the dark. Simulation of this spectrum yielded the following parameters: $g_{iso} = 2.0059$; $\langle a_N \rangle = 15.05$ G; $\langle a_H \rangle = 3.11$ G; $a = 0.432$; $b = -0.04$; $c = 0.075$. We assigned this spectrum to the nitroxide expected to be formed by trapping G• with PBN (Scheme 2). The coupling of the electron spin to the core spin of the nitrogen atom ($I = 1$) splits the signal into a triplet, while the β-H ($I = {}^1/_2$) splits the triplet further into doublets.

In the dark this nitroxide is stable for hours, but it decomposes upon irradiation. The decomposition leads to a secondary radical with a spectrum consisting of a triplet ($g_{iso} = 2.0056$, $\langle a_N \rangle = 17.17$ G), as shown in Figure 3B. The striking feature of this spectrum is that it is just a triplet, indicating that the second nitroxide has no β-H. Exposure of the first nitroxide to sunlight for 10 h leads to the decrease of the intensity of spectrum 3A, while at the same time the spectrum of the secondary nitroxide (spectrum 3B) grows in, resulting in a superposition of the two spectra (spectrum 3C). Additionally, another very weak signal may develop over this time scale. Because the two nitroxide signals increase and decrease in a correlated fashion, it is likely that the first nitroxide is converted into the second nitroxide upon exposure to light. The spectral features of the secondary nitroxide resemble those for di-*tert*-butyl nitroxide.

The site at which the guanine radical is trapped is proposed to be either the C4 or C5 position on guanine (Scheme 2), since no further hydrogen or nitrogen hyperfine coupling could be resolved. These positions were also the sites proposed in spin-trapping experiments on the guanosine nucleotide by MNP.[26] Furthermore, mechanisms for the formation of irreversible oxidation products of guanine often invoke addition to the guanine radical at the C4 position.[29]

Once again, we detected no signal in parallel trapping experiments carried out using poly(dA-dT) or poly(dI-dC) rather than poly(dG-dC) as substrate. This result further supports our assertion that, in the flash-quench experiment, the guanine radical within the DNA duplex is being formed selectively.

Guanine Radical Detection in Mixed-Sequence DNAs. To explore in more detail the selectivity of base oxidation, we carried out flash-quench experiments probed by EPR spectroscopy using two heteropolymeric DNAs: a 13-mer duplex containing a single guanine and calf thymus DNA, which has a GC content of 42%. Figure 4 shows the spectrum obtained upon irradiation of $[Ru(phen)_2dppz]^{2+}$ bound to the 13-mer duplex with $[Co(NH_3)_5Cl]^{2+}$ as quencher. In this case, a signal is apparent which possesses the same g_{iso} value, width, line

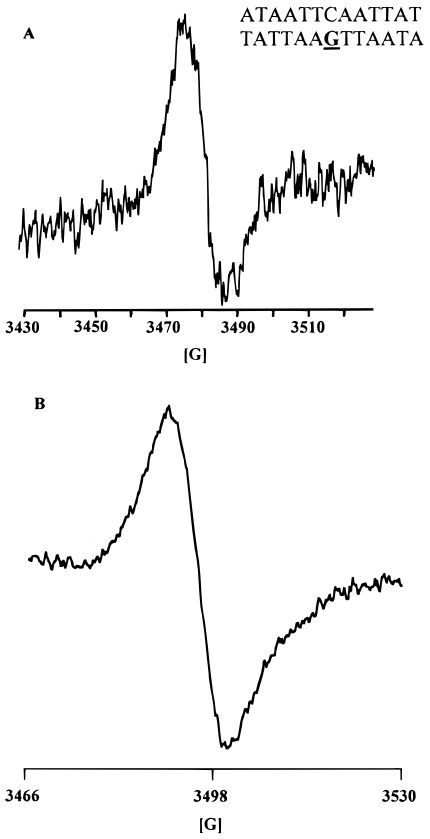


Figure 4. (A) EPR signal obtained during irradiation of an argon-saturated sample, containing a 13 base pair DNA duplex (7 mM) with a single GC base pair, 0.7 mM $[Ru(phen)_2(dppz)]^{2+}$, and 0.7 mM $[Co(NH_3)_5Cl]^{2+}$, $g_{iso} = 2.0048$, and $\triangle = 45$ G. The EPR settings and irradiation conditions were the same as for the spectrum in Figure 1(A). (B) EPR signal obtained during irradiation of a continuous flow of an argon-saturated sample, containing 40 mM sonicated calf thymus, 6 mM $[Ru(phen)_2(dppz)]^{2+}$, and 4 mM $[Co(NH_3)_5Cl]^{2+}$ in 10 mM tris buffer, pH 7. EPR settings: $T = 295$ K; microwave frequency 9.80 GHz; modulation frequency 100 kHz; modulation amplitude 10 G; microwave power 8 mW; conversion time 20.9 ms; receiver gain 2×10^5; number of scans 9. The flow rate was ~1 mL/min. $g_{iso} = 2.0048$, $\triangle = 45$ G, and half width at half-height = 8.5 G.

shape, and decay rate as described above with poly(dG-dC). We therefore also assign this signal to that of the guanine radical.

We next tested the flash-quench method to generate the guanine radical in calf thymus DNA. Preparing a sample with the equivalent concentrations as for poly(dG-dC) resulted in a weak signal, but increasing the Ru^{2+} intercalator concentration yielded the same EPR signal as with poly(dG-dC) and of similar order of magnitude in intensity. The need for higher concentrations is understandable given the lower G content of calf thymus DNA. The ready availability of calf thymus DNA enabled us to perform the EPR experiments within a flow cell. By doing so, we were able to increase the signal-to-noise ratio ~10-fold, leading to the signal in Figure 4b with the same g value and halfwidth as with poly(dG-dC). The signal appears now to be more symmetrical. The remaining asymmetry may be due to the slow tumbling of the DNA. It was not possible to resolve any hyperfine coupling even with a modulation amplitude of 0.1 G. This may be due to superposition of hyperfine couplings of guanine radicals in different environments and to the slow tumbling of the DNA which leads to considerable line broadening.

Spin-trapping of the radical generated in both mixed-sequence DNAs with PBN yielded the same nitroxide spectra as seen

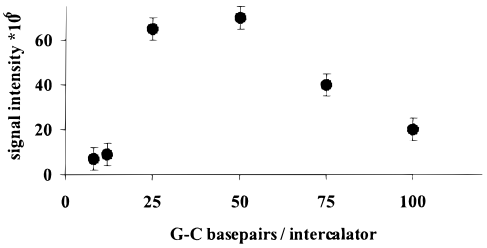


Figure 5. Plot of the double integrated EPR signal intensity versus the ratio of G−C base pairs/$[Ru(phen)_2(dppz)]^{2+}$ intercalator. Samples each contained 7 mM poly(dG-dC) and 0.7 mM $[Co(NH_3)_5Cl]^{2+}$ in addition to $[Ru(phen)_2(dppz)]^{2+}$. EPR signals were recorded during irradiation.

with poly(dG-dC). These results support further the assertion that G• is generated and trapped in all three cases and that it is the G• that is being formed selectively. It is noteworthy that none of the spectra of the trapped radicals show a M_I-dependent line broadening that one might expect for a large macromolecule in slow motion with strong nitrogen coupling.[19] The parameter c, which simulates the line broadening caused by slow tumbling, is ≈0.08 in all three cases. In contrast, EPR spectra of MNP-trapped X-irradiated DNA have shown a more pronounced M_I dependence, but the extent varied significantly depending upon the DNA or RNA examined.[26] The small M_I dependence in the experiments here may indicate an extrahelical orientation for the oxidized guanine after it is trapped by the bulky PBN. This would lead to higher degree of motional freedom for the nitroxide and therefore to a small M_I dependence. In the case of MNP, the resulting nitroxide may be small enough to fit within the double helix, resulting in a smaller degree of rotational freedom.

Influence of the Ru^{2+} Intercalator Concentration. To begin to probe any possible interaction of radicals within the DNA duplex, the concentration of radicals was increased systematically by increasing the Ru^{2+} intercalator concentration. For the experiments described thus far, the ratio of DNA base pairs/Ru^{2+} was 100:1. Upon increase of the Ru^{2+} intercalator concentration, the average separation between intercalators and hence radicals formed would be expected to decrease; this assumes the noncooperative binding of intercalators on the helix.[30]

Solutions containing poly(dG-dC) and $[Co(NH_3)_5Cl]^{2+}$ with increasing concentrations of Ru^{2+} intercalator were each irradiated and the EPR signals quantitated. Figure 5 shows a plot of the double integrated signal intensity as a function of DNA base pair/intercalator ratio. As the loading of the intercalator increases on the helix from 100:1 base pairs/Ru^{2+} to 50:1 base pairs/Ru^{2+}, the signal intensity is seen to increase, saturating at 25:1 base pairs/Ru^{2+}. However, increasing the loading still further until 8:1 leads to a significant decrease in signal. It is noteworthy that luminescence studies indicate that $[Ru(phen)_2dppz]^{2+}$ saturates in binding at a loading of approximately three base pairs/Ru^{2+}.[31]

Given that the decreased signal at loadings of greater than four Ru^{2+}/100 base pairs likely does not reflect decreased binding of the intercalator on the helix, the decrease in signal intensity we observe at these ratios might reflect instead a strong antiferromagnetic exchange interaction between radicals being generated. This exchange interaction would be proposed to begin to arise over an average radical separation of approximately 20 base pairs; this does not mean necessarily that there is a strong exchange interaction over the full 20 base pairs but rather that, statistically, there is a significant population of G•−G• distances below 20 base pairs which could yield strong antiferromagnetic

coupling. In the order from 50:1 to 25:1 a broadening of the signal by 3 G is observable, as one would expect for a rise of an exchange coupling. Spin-relaxation effects exerted by the higher Ru^{2+} concentration cannot account for the decreasing signal intensity since line broadening was accounted for by double integration and the microwave power was below saturation for all Ru^{2+} concentrations.

Clearly this proposal of strong antiferromagnetic exchange between radicals in DNA requires further experiments with covalently bound intercalators in assemblies where the $G^{\bullet}-G^{\bullet}$ distances are varied systematically, and such studies are underway. It is intriguing, however, to note that the saturation in signal intensity at a ratio of 1:25 in our experiment is reminiscent of earlier results. Symons and co-workers[32] examined γ-irradiated frozen DNA samples in which primarily thymine and cytosine radicals were formed. In the presence of the intercalator, mitoxantrone, electron transfer from the thymine and/or cytosine radicals to mitoxantrone occurred. By monitoring the EPR signal intensity of the mitoxantrone radical anion, they found a saturation of signal intensity at an intercalator to G/C base pair ratio of 1:30. It is also notable that, in photoinduced luminescence quenching experiments as a result of electron transfer between metallointercalators noncovalently bound to DNA, the quenching yield increased linearly with increased metal quencher until saturating at a loading of approximately 1 intercalator/20 base pairs; the rate of recombination, measured using transient absorption was 10^{10} s^{-1} and did not vary significantly with loading.[33] Remarkably, it is over essentially the same regime where long-range DNA-mediated electron-transfer quenching occurred that we now observe a decrease in EPR signal, a possible indication of radical−radical interactions.

Summary and Implications. Guanine radicals in intact DNA duplexes have now been detected for the first time under biological conditions in aqueous solution by EPR spectroscopy. Moreover, guanine radicals may be detected in mixed-sequence DNA duplexes with high base selectively using the flash-quench methodology.

However, specifically, on the basis of energetics, why do we observe only guanine base radicals rather than a mixture of guanine and adenine radicals? Various possibilities arise. First there is the question of whether the Ru^{3+} intercalator, once formed, is sufficiently potent thermodynamically to oxidize adenine within DNA. A survey of measured reduction potentials for Ru^{3+}/Ru^{2+} of 1.6 V in acetonitrile versus 1.4 V for $A^{\bullet+}/A$ suggests that oxidation should be possible, but these values are based upon irreversible electrochemistry and were not carried out in the context of DNA.[16] Another possibility is that the adenine radical is indeed formed but it is quickly lost, either due to back-electron-transfer processes or due to subsequent quenching. Adenine radicals characterized by transient absorption spectroscopy have shown decay times of 10^8 M^{-1} s^{-1}.[34] It has also been proposed that the formation of $G^{\bullet}$ in the DNA helix is facilitated by rapid proton transfer from $G^{\bullet+}$ to the complementary C, yielding the lower energy $G^{\bullet}$; if formed, $A^{\bullet+}$ has no analogous proton-coupled trapping reaction available.[35]

It is interesting also to consider the preponderance of guanine radicals and notable absence of adenine radicals we detect in the context of some current mechanisms for charge migration through DNA which are being debated. In particular, Giese and co-workers have proposed that electron holes migrate in the DNA bridge in hops from guanine to guanine bases;[36] guanines are proposed as the stepping stones owing to their low oxidation potential compared to other bases in the bridge. Other studies have suggested that the sequence dependence in yield of DNA-mediated charge transport may be more complex and that sequence-dependent structural issues are critical as well as sequence composition.[4,37] Also recently, a polaron-assisted hopping mechanism has been proposed, in which structural distortions propagated through the helix are important and some extent of delocalization within the polaron may occur; the length and sequence characteristics of these proposed polarons have not yet even been suggested, however.[37]

The interaction of radicals within DNA will be interesting to consider in the context of the various proposed mechanisms. Indeed, if possible long-range antiferromagnetic exchange coupling of radicals within DNA is established, then it would require that mechanistic descriptions of electronic interactions within DNA may need to be refined still further. Certainly our application of EPR spectroscopy to examine radical reactions and interactions within DNA will offer a powerful new probe to characterize this chemistry.

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